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Mechanistic studies on the synergistic cytotoxicity of the nucleoside analogs gemcitabine and clofarabine in multiple myeloma: Relevance of p53 and its clinical implications

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Abstract

Hematopoietic stem cell transplantation (HSCT) is an established treatment for multiple myeloma (MM), a plasma cell malignancy. To identify an improved pretransplant conditioning regimen, we investigated the cytotoxicity of gemcitabine (Gem) and clofarabine (Clo) combinations toward MM cell lines and patient cell samples. A strong synergism of the two nucleoside analogs, when combined at their approximate IC₁₀ concentrations, was observed. This synergism could be partly due to the observed Gem-mediated phosphorylation and activation of deoxycytidine kinase, resulting in enhanced phosphorylation of Gem and Clo. Their cytotoxicity correlated with a robust activation of the DNA damage response pathway. [Gem+Clo] decreased the mitochondrial membrane potential with a concomitant release of proapoptotic factors into the cytoplasm and nucleus and the activation of apoptosis. Exposure of MM cells to [Gem+Clo] also decreased the level of ribosomal RNA (rRNA), which might have resulted in nucleolar stress, as reported previously, and caused a p53-dependent cell death. A reduction by approximately 50% in the cytotoxicity of Gem and Clo was observed in the presence of pifithrin, a p53 inhibitor. Furthermore, MM cell lines with mutant p53 exhibited greater resistance to Gem and Clo, supporting a role for the p53 protein in these cytotoxic responses. Our results provide a rationale for clinical trials incorporating [Gem+Clo] combinations as part of conditioning therapy for high-risk patients with MM undergoing HSCT.

Multiple myeloma (MM) is a malignancy of plasma cells that accumulate in the bone marrow and interfere with the production of normal blood cells. The median age at diagnosis is approximately 70 years, and the disease accounts for approximately 10% of hematologic malignancies [1]. Chemotherapeutic interventions for MM include proteasome inhibitor (bortezomib) and immunomodulatory therapy (thalidomide and lenalidomide). Recently, advances in autologous and allogeneic hematopoietic stem cell transplantation (HSCT) have further improved the prognosis for patients with MM; the differential application of different

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pretransplant regimens according to age has improved the survival rate [2–6]. However, relapses continue to shorten survival and remain a big challenge for clinical investigators.

The efficacy of pretransplant regimens used with HSCT has a major role in the success of this form of treatment. Whether reduced-intensity conditioning or high-dose chemotherapy regimens are more efficacious for MM patients undergoing HSCT remains unresolved and requires further study. Reduced-intensity conditioning for allogeneic HSCT has not been generally associated with improved progression-free survival or overall survival [5], which suggests a need to revisit high-dose chemotherapy preparative regimens for high-risk patients with MM. The most commonly used preparative agents, usually in combinations, are melphalan, busulfan, and cyclophosphamide; however, multiple other agents such as etoposide, cytarabine, fludarabine, vincristine, doxorubicin, dexamethasone, bortezomib, thalidomide, and lenalidomide have also been used [7]. Limited studies have been performed with the nucleoside analog gemcitabine (Gem), either as a single agent or as part of a combination with other drugs, in a pretransplant conditioning regimens for patients with MM [8,9]. However, Gem has been shown to improve responses or survival when used as part of pretransplant regimens for leukemia and lymphoma patients [10–15]. In a nontransplant setting, a favorable activity of combined Gem and paclitaxel has been observed in relapsed or refractory multiple myeloma [16], suggesting the benefit of a Gem-containing regimen.

Clofarabine (Clo) is another nucleoside analog with impressive antileukemia activity, and it has been shown to significantly improve outcomes for patients with acute myelogenous leukemia (AML), myelodysplastic syndrome, acute lymphocytic leukemia, and lymphoma when used in a transplant setting [17–21]. Based on the efficacy of Gem and Clo in the aforementioned hematologic malignancies, we hypothesized that their combination might provide synergistic cytotoxicity toward MM cells. We report in this preclinical study the cytotoxicity of Gem and Clo in both MM cell lines and patient-derived samples, and we propose possible mechanisms of the observed synergism. The results provide a mechanistic framework for designing both conventional therapy and improved high-dose conditioning regimens for MM patients undergoing HSCT.

Methods

Cell lines and drugs

The four cell lines used in this study were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI 1640 (Mediatech, Manassas, VA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA, USA) and 100 U/mL penicillin and 100 µg/mL streptomycin (Mediatech) at 37 °C in a humidified atmosphere of 5% CO₂. The cytogenetic characteristics of the four cell lines are listed in Table 1. Clofarabine (Clolar) was obtained from Genzyme Oncology (Cambridge, MA; 1 mg/mL solution) and diluted in RPMI 1640 medium prior to use, and gemcitabine (Eli Lilly, Indianapolis, IN) was dissolved in phosphate-buffered saline (PBS). Busulfan (Bu), melphalan (Mel), pifithrin- α , valinomycin, and cyclosporin A (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in dimethyl sulfoxide.

Cytotoxicity assay

Cell suspensions were aliquoted (100 µL of 2×10^5 cells/mL) into 96-well plates in the presence of drugs or solvent alone and continuously incubated at 37 °C for 48 hours. The cells were analyzed for cytotoxicity by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [22]. Graphical analyses including calculations of IC₁₀

values (the concentration of drug required for 10% growth inhibition) were done using Prism 5 (GraphPad Software, San Diego, CA, USA). Drug combination effects were estimated based on the combination index (CI) values [23] calculated using the CalcuSyn software (Biosoft, Ferguson, MO, USA). This program was developed based on the median-effect method: $CI < 1$ indicates synergy, $CI \approx 1$ is additive, and $CI > 1$ suggests antagonism.

Apoptosis assay

Cell death by apoptosis following a 48-hour drug exposure was determined by flow cytometric measurements of phosphatidylserine externalization [24] with the Annexin-V-FLUOS (Roche Diagnostics, Indianapolis, IN, USA) and 7-aminoactinomycin D (BD Biosciences, San Jose, CA, USA) kits using a Muse Cell Analyzer (EMD Millipore, Billerica, MA, USA). The extent of cleavage of poly(ADP-ribose) polymerase (PARP)-1 and caspases 3 and 8, determined by Western blot, was also used as an indicator of apoptosis.

Western blot analysis

Cells (0.5×10^6 /mL) were exposed to drugs or solvent for 48 h, collected by centrifugation, washed with cold PBS, and lysed with cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA). The protein concentrations were determined using a BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Proteins were resolved on polyacrylamide-SDS gels and blotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Western blot analyses were done by chemiluminescence using the Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore). Most of the antibodies, their sources, and other relevant information were described previously [25,26]. The antibody against deoxycytidine kinase (DCK) phosphorylated at Ser74 [27] was provided by Prof. F. Bontemps (Laboratory of Physiological Chemistry and Hormone and Metabolic Research Unit, Brussels, Belgium).

Analysis of mitochondrial membrane potential

Changes in the mitochondrial membrane potential were measured using a JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbo cyanine iodide) mitochondrial membrane potential detection kit (Cayman Chemical, Ann Arbor, MI, USA). Cells were exposed to drugs for 48 hours and 0.5-mL cell suspension was aliquoted into 5-mL tubes. Valinomycin (1 μ mol/L) was added to a positive control tube containing untreated cells and incubated at 37°C, 5% CO₂ for 1 hour. Diluted (1:10 with cell growth medium, 40 μ L) mitochondrial membrane potential-sensitive fluorescent dye JC-1 reagent was added to each tube, incubated at 37°C for 20 min, and immediately analyzed by flow cytometry as described by the manufacturer.

Isolation of cytosolic and mitochondrial fractions

Cells were exposed to drugs for 48 hours, collected by centrifugation, washed with ice-cold PBS, resuspended in buffer A (10 mmol/L HEPES [pH 7.6], 10 mmol/L KCl, 100 μ mol/L ethylenediaminetetraacetic acid [EDTA], 100 μ mol/L ethyleneglycoltetraacetic acid, 1 mmol/L dithiothreitol, 500 μ mol/L phenylmethylsulfonyl fluoride and proteinase inhibitor cocktail) and incubated on ice for 30 min. The cell membrane was disrupted by passing the cells ten times through a fine needle (27½ gauge). Cell homogenates were centrifuged at $800 \times g$ for 5 min at 4°C to pellet nuclei. The supernatant containing mitochondria and cytosolic extracts was further centrifuged at $12,500 \times g$ at 4°C for 8 min to pellet mitochondria, and the resulting supernatant was saved as the cytosolic fraction. The pellet (mitochondria) was washed with ice-cold buffer A and resuspended in ice-cold lysis buffer B (10 mmol/L HEPES [pH 7.6], 300 mmol/L KCl, 1 mmol/L EDTA, 0.5% Triton X-100, 5% glycerol, 1

mmol/L DTT and proteinase inhibitor cocktail). The cytosolic and mitochondrial extracts were further centrifuged at $15,000 \times g$ for 20 min at 4°C, and analyzed by Western blot.

Analysis of ribosomal RNA

Total RNA was isolated from cells exposed to drugs (for 48 hours) using RNazol (Life Technologies). Reverse transcription polymerase chain reaction (RT-PCR) was done using the OneStep RT-PCR Kit (Qiagen, Germantown, MD, USA). For preribosomal RNA (rRNA), a 30- μ L reaction mixture contained 1X reaction buffer, 1X buffer Q, 0.25 mmol/L deoxyribonucleoside triphosphate, 0.8 μ g RNA, 1.5 nmol/L primer 1, 1.5 nmol/L primer 2, and 1.2 μ L OneStep RT-PCR enzyme mix. Primers for pre-rRNA correspond to nt 5378-5396 (5'-TTGGATGGGTTTAGT-AGGC-3') and nt 5752-5771 (5'-CACGCCCTTCTTTCTCTCTC-3', GenBank Accession no. U13369), and the resulting amplified DNA included the 3' end of 18 S and the 5' end of internal transcribed spacer 1. The RT-PCR mixture for GAPDH (control) did not contain buffer Q. The primers for GAPDH correspond to nt 33-52 (5'-GGTGAAGGTCGGAGTCAACG-3') and nt 510-529 (5'-CAAAGTTGTCATGGATGACC-3', GenBank Accession no. BC023632). An ABI 7500 Real Time PCR System (Applied Bio-systems, Foster City, CA, USA) was used to do reverse transcription at 50°C for 30 min, followed by 95°C heating for 15 min to denature reverse transcriptase and activate HotStarTaq DNA polymerase. The succeeding 35 cycles of PCR included denaturation at 94°C for 0.5 min, and annealing and extension at 60°C for 1 min. A final extension reaction was done at 72°C for 10 min. The RT-PCR products for pre-rRNA (8.5 μ L) and GAPDH (1.5 μ L) were mixed with 2 μ L 6X loading buffer and loaded onto an 0.8% agarose gel. After electrophoresis and staining with ethidium bromide, the gel was analyzed using the G:BOX EF² (Syngene, Frederick, MD, USA) and its GeneSys software.

Flow cytometric analysis of rRNA was done using H929 cells exposed to drugs for 48 hours. Cells were harvested by centrifugation, washed with Buffer PTB (PBS, 0.1% Tween 20, 1% bovine serum albumin), and fixed with 1% paraformaldehyde in PBS for 10 min at 37°C. Cells were centrifuged at $1200 \times g$ for 6 min at room temperature and resuspended in PBS. The cell membrane was permeabilized with 70% ethanol at -20°C overnight followed by centrifugation at $1200 \times g$ for 6 min at 4°C and washing with Buffer PTB. Blocking was done using 10% FBS in Buffer PTB overnight at 4°C. Cells were then centrifuged as described earlier and tumbled overnight at 4°C with anti-rRNA antibody Y10 b (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:200 in Buffer PTB. Cells were washed three times with Buffer PTB, resuspended in 1:2000 diluted anti-mouse IgG antibody conjugated to Alexa Fluor 488 (Life Technologies) and tumbled in the dark at 4°C for 1 hour. Cells were washed three times with Buffer PTB and stained with 5 μ g/mL propidium iodide in Buffer PTB for 30 min before analysis by flow cytometry.

Patient cell samples

Bone marrow aspirates from patients with MM were collected after obtaining written informed consent. Mononuclear cells were purified using lymphocyte separation medium (Mediatech), and CD138-positive cells were separated using the EasySep Human CD138 Positive Selection Kit (StemCell Technologies, Vancouver, Canada). The purified cells were incubated in suspension in RPMI 1640 supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μ g/mL streptomycin with the indicated drugs. Cells were analyzed after 48 hours of incubation. All studies were performed according to a protocol approved by the Institutional Review Board of the University of Texas MD Anderson Cancer Center, in accordance with the Declaration of Helsinki.

Results

Gemcitabine and clofarabine provide synergistic cytotoxicity toward multiple myeloma cell lines

Our previous preclinical and clinical studies have shown the cytotoxicity of various combinations of nucleoside analogs and DNA alkylating agents in AML and lymphoma cells [9,17,25,26,28]. We hypothesized that these drugs would have similar cytotoxicity toward MM cells. NCI-H929 and MM.1R cells were continuously exposed for 48 hours to predetermined IC_{10} - IC_{20} of the drugs, singly or in combination, and analyzed for cytotoxicity by MTT and annexin V assays. Exposure of both cell lines to the indicated concentrations of Bu, Mel, Gem and Clo inhibited cell proliferation by a maximum of 10% and increased apoptosis by a maximum of 5%, as indicated by annexin V-positive cells (Fig. 1). Combination of one nucleoside analog (Gem or Clo) with a DNA alkylating agent (Bu or Mel) did not significantly further decrease cell proliferation nor increase apoptosis. However, exposure of NCI-H929 cells to a combination of 3.5 nmol/L Gem and 250 nmol/L Clo decreased cell proliferation to 47% and increased annexin V-positive cells to 45% (Fig. 1A). More substantial effects were observed when MM.1R cells were exposed to [Gem+Clo] combination. Cell proliferation decreased to 25%, and annexin V-positive cells increased to 61% (Fig. 1B). Addition of Bu or Mel to the [Gem+Clo] combination did not greatly alter the cytotoxicity of the two nucleoside analogs toward the two myeloma cell lines. Analysis of the cytotoxicity of [Gem+Clo] combination using the method of Chou and Talalay [23] resulted in combination indexes of 0.3–0.7 for NCI-H929 cells and 0.1–0.2 for MM.1R cells, suggesting strong synergism of the nucleoside analogs (Fig. 1C and D).

Because fludarabine (Flu) and cladribine (Clad) are two other purine analogs used against lymphoid malignancies, we also examined their cytotoxicity in combination with Gem. Neither the combination of [Gem+Flu] nor [Gem+Clad] showed a degree of synergism comparable to [Gem+Clo] in NCI-H929 and MM.1R cells (data not shown). However, the three-drug combination of 3.5 nmol/L Gem, 7 μ mol/L Flu, and 100 nmol/L Clad provided equivalent, synergistic cytotoxicity compared with 3.5 nmol/L Gem plus 250 nmol/L Clo in NCI-H929 cells.

[Gem+Clo] combination activates the DNA-damage response signaling network

We previously showed activation of the DNA-damage response (DDR) in AML and lymphoma cells exposed to combinations of nucleoside analogs and DNA alkylating agents [25,26,28]. To determine whether the [Gem+Clo] combination has a similar mechanism of cytotoxicity, MM cells exposed to drugs were analyzed for changes in selected key DDR protein levels and modifications. Exposure of NCI-H929 cells to different drug combinations resulted in the phosphorylation of ATM at Ser1981, with by far the highest signal being obtained in cells exposed to combinations containing [Gem+Clo] (Fig. 2A). This observation correlates with the phosphorylation of known ATM kinase substrates such as histone 2AX, KAP1, and p53 (Fig. 2A).

A widely used indicator of DDR is the phosphorylation of histone 2AX, commonly called γ -H2AX [29]. Figure 2A shows the highest level of γ -H2AX in cells exposed to [Gem+Clo]. The addition of Bu or Mel to this drug combination did not further increase the level of γ -H2AX. Another ATM substrate is p53 protein, which is involved in cell cycle checkpoint activation, DNA repair regulation, and apoptosis. Exposure of NCI-H929 cells to different drug combinations increased both the level of p53 protein and its Ser15-phosphorylated form, with the highest signals being in cells exposed to [Gem+Clo], again with or without Bu or Mel (Fig. 2A). These results suggest that the synergistic cytotoxicity of [Gem+Clo] is associated with a profound activation of the DDR.

Because the DDR is accompanied by a concomitant chromatin remodeling to facilitate DNA repair, we also examined the ATM-mediated phosphorylation of the transcriptional coregulator KRAB-associated protein (KAP1) at Ser824 [30]. Exposure of NCI-H929 cells to [Gem+Clo] increased the phosphorylation of KAP1 (Fig. 2A), further indicating activation of a comprehensive DDR.

In addition to ATM, activation of the DDR can involve other kinases such as ATR (ATM- and RAD3-related). Examination of the phosphorylation of ATR at Ser428 in control cells and in cells exposed to various drug combinations did not reveal any dramatic differences (Fig. 2A), suggesting a possible lack of involvement of ATR in the cytotoxicity of [Gem+Clo].

[Gem+Clo] combination triggers apoptosis

The observed extensive DDR can reflect an underlying complex genomic injury and commits the cells to apoptosis. As indicated by an increase in the proportion of annexin V-positive cells (Fig. 1), a considerable proportion of NCI-H929 myeloma cells underwent apoptosis when exposed to [Gem+Clo]. This observation is supported by the extensive cleavage of PARP-1, activation (by cleavage) of caspase 8 and 3, and downregulation of the prosurvival proteins BCL-XL and c-MYC (Fig. 2B).

To further understand the mechanism of drug-mediated apoptosis, changes in the mitochondrial membrane potential ($\Delta\psi$) were determined. A decrease in $\Delta\psi$ is known to result in the release of proapoptotic factors from the mitochondria into the cytoplasm [31]. We probed these events using JC-1 reagent, a lipophilic cationic dye that enters the mitochondria and forms aggregates with intense red fluorescence. A decrease in $\Delta\psi$ causes translocation of JC-1 to the cytoplasm, where it is converted to its monomeric form that emits a green fluorescence. As a positive control, a 1-hour exposure of MM.1R cells to 1 μ mol/L valinomycin, a K^+ -selective ionophore, followed by addition of JC-1 and flow cytometric analysis, showed approximately 85% and 15% green and red fluorescence, respectively, suggesting a significant decrease in $\Delta\psi$ (Fig. 3A). Exposure of cells to 3 nmol/L Gem or 250 nmol/L Clo for 48 hours increased green fluorescence from 5% to 15% or 10%, respectively; combination of Gem and Clo increased the green signal to 73%. Similar results were observed when NCI-H929 cells were exposed to [Gem+Clo], suggesting that the two nucleoside analogs activate apoptosis by decreasing mitochondrial membrane potential.

The observed drug-mediated decrease in $\Delta\psi$ was expected to cause leakage of mitochondrial proteins into the cytoplasm. We therefore sought to determine changes in the level of proapoptotic factors in the cytosolic fraction after drug exposure. As shown in Figure 3B, the cytosolic levels of proapoptotic BAX, AIF, cytochrome c, and BID were indeed increased, with by far the greatest increase being in cells exposed to [Gem+Clo]. These proteins are known to be involved in a cascade of events leading to the activation of caspases (Fig. 2B). In fact, inhibition of [Gem+Clo]-mediated mitochondrial permeability with 2 μ mol/L cyclosporin A [32] inhibited the cleavage of PARP-1, caspase 3, and caspase 8 (Fig. 3C), further supporting the hypothesis that the cytotoxicity of [Gem+Clo] in myeloma cells involves decreased $\Delta\psi$. As anticipated, the cyclosporin A treatment did not result in a decrease in $\Delta\psi$ (data not shown).

[Gem+Clo] combination results in decreased cellular levels of DNA repair proteins

The activated forms of nucleoside analogs are known to inhibit ribonucleotide reductase, resulting in a decreased deoxynucleotide pool and inhibition of DNA repair [33,34]. Nucleoside analogs can also inhibit the repair of damaged DNA through other mechanisms.

As shown in Figure 4, we looked for possible changes in the cellular levels of the BRCA1, XRCC1, Ku80, Rad51, NBS1, MRE11, FANCA, and FANCD2 DNA-repair proteins. These levels were all diminished at the end of a 48-hour exposure to [Gem+Clo]. The decrease in some, if not all, of these proteins could be attributed to caspase-mediated protein cleavage as shown for PARP-1 (Fig. 2B and 3C) and elsewhere for BRCA1 [35], Ku80 [36], and Rad51 [37]. Indeed, DNA repair pathways are well known targets for activated caspases [38]. It is also possible that a component of the degradation observed at 48 hours reflects drug-mediated effects on DNA repair proteins that precede caspase activation. Discriminating these mechanisms will require more detailed studies including the effect of blocking caspase activity.

Gem induces phosphorylation of deoxycytidine kinase

Deoxycytidine kinase catalyzes the initial activation and phosphorylation of Gem and Clo [39,40]. Its enzymatic activity is increased by ATM-mediated phosphorylation at Ser74, suggesting a possible role for the DNA damage response in this process [41,42]. Other factors that might contribute to DCK activation are not known. How normal nucleotides and nucleoside analogs affect the phosphorylation of DCK has not been reported. We therefore sought to examine the effects of Gem and Clo on the phosphorylation of DCK. Exposure of MM.1R cells to 250 nmol/L Clo did not change the level of phosphorylated DCK; exposure to 3 nmol/L Gem or [Gem+Clo] significantly increased Ser74 phosphorylated DCK levels (Fig. 4). These results suggest that the observed synergistic cytotoxicity of [Gem+Clo] may be partially due to Gem-mediated activation of DCK resulting in enhanced phosphorylation of both nucleoside analogs. This could be important for optimized sequencing of the agents in a clinical therapeutic setting.

The cytotoxic activity of Gem, Clo, and [Gem+Clo] combination against myeloma cells is partially p53 dependent

During the analysis of Gem and Clo cytotoxicity toward MM cell lines, we observed that the RPMI 8226 and U266B1 cell lines were less sensitive to these drugs. Dose-response analysis showed an IC₅₀ of 10 nmol/L and of 20 nmol/L Gem for MM.1R and NCI-H929 cells, respectively, compared with greater than 100 nmol/L for both RPMI 8226 and U266B1 cells (Fig. 5A). Similarly, the IC₅₀ values for Clo were 400 nmol/L and 900 nmol/L for NCI-H929 and MM.1R cells, respectively, and greater than 10 μmol/L Clo for the other two cell lines (Fig. 5B). Cytogenetic analysis of these four cell lines has shown that NCI-H929 and MM.1R cells harbor wild type *p53*, whereas RPMI 8226 and U266B1 cells have mutated *p53* [43], consistent with our immunoblot analysis showing the presence of a lower-molecular-weight protein recognized by the anti-p53 antibody used in the study (Fig. 5C).

To determine whether pharmacologic inhibition of the transactivation-function of p53 would abrogate the cytotoxicity of the combination of [Gem+Clo], NCI-H929 cells were preexposed to 20 μmol/L pifithrin [44]. Whereas [Gem+Clo] exposure for 48 hours resulted in 60% annexin V-positive cells, preexposure to pifithrin- prior to [Gem+Clo] decreased annexin V-positive cells to 32% (Fig. 5D). This partial reversal of [Gem+Clo] cytotoxicity by pifithrin- is consistent with an observed decrease in PARP-1 cleavage, phosphorylation of histone 2AX, and levels of p21 and PUMA (Fig. 5E). Overall, these results suggest an important role for p53 in the cytotoxicity of [Gem+Clo] on MM cells.

[Gem+Clo] combination inhibits rRNA production

Nucleoside analogs are known to inhibit retroviral RNA replication [45] and RNA synthesis in mammalian cells [46]. Adenosine analogs have been shown to inhibit polyadenylation of mRNA [47]. If the [Gem+Clo] combination inhibits RNA production, we hypothesized that it might have greater effects on the production of rRNA, which is transcribed from multiple

copies of the rRNA gene. Reverse transcription-PCR analysis shows approximately 40% inhibition of pre-rRNA synthesis relative to transcription of GAPDH (Fig. 6A). Flow cytometric analysis using an anti-body against rRNA showed a decrease in fluorescence signal of cells exposed to [Gem+Clo] compared with control (Fig. 6B), consistent with the inhibition of prerRNA production (Fig. 6A). Immunoblot analysis of proteins involved in pre-rRNA transcription and processing showed decreased levels of UBF [48], p120 [49], and treacle [50] but an increase in the level of RPA135 (Fig. 6C), a subunit of RNA polymerase I that transcribes pre-rRNA genes [51].

[Gem+Clo] combination has similar synergistic effects in patient-derived multiple myeloma cells

To assess the potential clinical implications of the observed synergism of Gem and Clo in myeloma cell lines, we isolated CD138-positive cells from bone marrow aspirates from patients with a diagnosis of MM. Cells were exposed to Gem and Clo, singly or in combination. Immunoblot analysis showed increased phosphorylation of histone 2AX in cells exposed to [Gem+Clo], suggesting robust activation of the DDR pathway. The elevated baseline level of γ -H2AX in Pt 08 could be due to the drugs the patient was taking at the time of bone marrow sampling. The synergistic activity of [Gem+Clo] in these patient samples is further supported by an increased expression of p53, a DDR protein involved in cell-cycle arrest and apoptosis (Fig. 7).

Discussion

The development of an improved conventional therapy as well as a safe and more efficacious conditioning regimen for high-risk MM patients undergoing HSCT is of utmost importance. The previously reported anti-neoplastic activities of nucleoside analogs both in non-HSCT and HSCT studies in leukemia and lymphomas suggested that these drugs might also be effective in MM. We present in this study evidence of a strong synergistic cytotoxicity of Gem and Clo already at low drug concentrations (IC_{10} - IC_{20}) in MM cell lines and in cells derived from patients with MM. The observed cytotoxicity of the two nucleoside analogs could be attributed to at least five interrelated mechanisms as illustrated in Figure 8: (1) Gem-mediated activation/phosphorylation of DCK, (2) inhibition of DNA synthesis and DNA repair [34,52,53], (3) nucleolar stress through inhibition of rRNA production, (4) decrease in the γ -H2AX, and (5) induction of apoptosis.

Gem and Clo are pro-drugs that are activated by phosphorylation. This reaction is catalyzed by DCK, an enzyme that assumes a more active conformation when phosphorylated at Ser74, which facilitates nucleoside binding and release [54]. We observed that Gem, but not Clo, induces phosphorylation of DCK at Ser74 (Fig. 4) by a mechanism yet to be determined. This Gem-mediated phosphorylation of DCK would suggest activation of the enzymatic activity of DCK with a concomitant enhancement of the phosphorylation of both Gem and Clo. Increased phosphorylation of the drugs can result in increased inhibition of ribonucleotide reductase [52] with a consequential decrease in the deoxynucleotide pool including dCTP, a strong feedback inhibitor of DCK. The resulting lower dCTP concentration could alleviate its feedback inhibition of DCK and further increase the concentration of phosphorylated/activated Gem and Clo [41]. In fact, exposure of lymphoma cell lines to Gem before adding Clo is more cytotoxic than the reversed order of drug exposure (data not shown), consistent with Gem-mediated activation of DCK. The relevance of DCK in the cytotoxicity of Gem is consistent with the previously reported inactivation of DCK in Gem-resistant cancer cells [55,56].

The presumed Gem-mediated increase in the activity of DCK would subsequently increase the cellular concentrations of the diphosphate forms of Gem and Clo, which are known to

inhibit ribonucleotide reductase [33,34] and consequently decrease the concentrations of normal deoxy-nucleotides. Such a scenario would further increase the incorporation of the nucleoside analogs into growing nascent DNA strands, resulting in augmented inhibition of DNA synthesis and possibly DNA repair and an increase in the level of DNA strand breaks. This drug-mediated induction of DNA breaks is consistent with the observed activation of the ATM pathway and phosphorylation of its downstream substrates, including histone 2AX and p53 (Fig. 2A) as well as DCK itself (Fig. 4) [42]. Phosphorylation of p53 can trigger the accumulation of BAX in the cytosol (Fig. 3B) and its translocation to the mitochondrial membrane to decrease transmembrane potential $\Delta\psi_m$ (Fig. 3A), induce mitochondrial permeability, and release proapoptotic factors, including cytochrome c (Fig. 3B). The released cytochrome c would then initiate the cascade of events leading to the activation of caspases (Fig. 3C) and programmed cell death [57].

Apoptosis-inducing factor (AIF) was also released from the mitochondria in cells exposed to [Gem+Clo]. We observed an accumulation of AIF in the cytoplasm (Fig. 3B) and the nucleus (data not shown). AIF has been shown to induce chromatin condensation and DNA fragmentation [58]. The AIF-mediated increase in DNA breaks can then trigger further release of cytochrome c and AIF from the mitochondria, contributing to the increased cytotoxicity of [Gem+Clo].

Gem and Clo have direct deleterious effects on the mitochondria. Gem is phosphorylated by thymidine kinase in the mitochondria [59], suggesting that it might inhibit mitochondrial DNA synthesis. The direct binding of Clo triphosphate metabolites to proteins in the mitochondrial membrane is known to decrease $\Delta\psi_m$ and allow release of proapoptotic factors, which are important in the formation of the apoptosome [60]. In fact, Clo alone increased the level of cytochrome c in the cytoplasm (Fig. 3B). These direct effects of Gem and Clo on the mitochondria might account for part or all of that component of their cytotoxicity that is independent of p53 (Fig. 8).

The ability of [Gem+Clo] to inhibit rRNA biogenesis (Fig. 6), which causes nucleolar stress [61], might also contribute to the observed activation of the apoptotic pathway by these drugs. In response to such stress, some nucleolar proteins translocate to the nucleoplasm, bind to MDM2, and block p53 ubiquitination and degradation. The stabilized p53 protein then induces cell cycle arrest and apoptosis [61], which is consistent with the observed increased level of phosphorylated and total p53 in cells exposed to [Gem+Clo] (Fig. 2A, illustrated in Fig. 8).

The pivotal role of p53 in the cytotoxicity of [Gem+Clo] (Fig. 8) is shown by the significant, albeit partial, reversal of this cytotoxicity in the presence of pifithrin- α (Fig. 5D and E). The inability of pifithrin- α to completely reverse the cytotoxicity of [Gem+Clo] suggests possible contributions of p53-independent pathways (discussed earlier) or an inability of pifithrin- α to inhibit p53 activity completely. This observation raises a potential relevance of determining p53 status when assessing clinical responses of patients with MM to a [Gem+Clo]-based treatment.

In summary, our study suggests that the synergistic anti-myeloma activity of [Gem+Clo] combinations can be mechanistically attributed to the activation of DCK and concomitant increase in the phosphorylated form of Gem and Clo, which exacerbates the inhibition of a number of cellular pathways including DNA synthesis (nucleus and mitochondria), DNA repair, and rRNA production (Fig. 8). Most of these events generate signals that converge to stabilize and activate proapoptotic p53 protein and cause mitochondrial leakage, resulting in programmed cell death. Although the clinical efficacy of a [Gem+Clo]-based regimen used in clinical therapy of MM remains to be investigated, their cytotoxicity toward CD138-

positive cells isolated from patients with MM provides a mechanistic impetus for evaluating [Gem+Clo]-based combinations as a part of cytoreductive treatment programs in patients with MM.

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Figure 1.

Cytotoxicity of combinations of nucleoside analogs and DNA alkylating agents toward multiple myeloma cell lines. NCI-H929 and MM.1R cells were exposed continuously to drugs alone or in combination for 48 hours and analyzed using MTT and annexin V (Ann V) assays (**A** and **B**). Synergism of cytotoxicity of gemcitabine and clofarabine was determined by calculating combination indexes (**C** and **D**) according to the method of Chou and Talalay [23].

Figure 2.

Combinations of Gem and Clo activate elements of the DNA damage response (**A**) and apoptosis (**B**) pathways. NCI-H929 cells were continuously exposed to drugs alone or in combination for 48 hours, and changes in the expression or phosphorylation status of proteins involved in these pathways were determined using immunoblot analysis.

Figure 3.

Drug-mediated effects on the mitochondrial membrane potential. MM.1R cells were exposed to drugs for 48 hours and analyzed using (A) the JC-1 membrane potential probe as described in the Methods section, and (B) Western blot of the cytosolic and mitochondrial fractions for levels of proapoptotic proteins. (C) Cells were exposed to Gem and Clo in the absence or presence of cyclosporin A (Cyclo A), a known inhibitor of mitochondrial permeability, and analyzed for biomarkers of apoptosis using Western blot. COX IV or β -actin, or both, were probed as a loading control.

Figure 4. Effects of nucleoside analogs on the cellular levels of DNA repair proteins and on the phosphorylation of deoxycytidine kinase (DCK). MM.1R cells were exposed to Gem and Clo alone or in combination for 48 hours and analyzed using Western blot.

Figure 5.

Dependence of the cytotoxicity of gemcitabine (Gem) and clofarabine (Clo) on p53 status. MM cell lines were exposed to different concentrations of Gem or Clo for 48 hours and analyzed by MTT assay (**A** and **B**). p53 levels in these MM cell lines were analyzed using Western blot (**C**). NCI-H929 cells were exposed to 20 μ mol/L pifithrin- (PF) for 24 hours preceding exposure to 3.5 nmol/L Gem and 250 nmol/L Clo for 48 hours and analyzed for biomarkers of apoptosis using flow cytometry (annexin V; Ann V) (**D**) or Western blot (**E**).

Figure 6. Drug-mediated inhibition of rRNA production. NCI-H929 cells were exposed to drugs for 48 hours and analyzed for the level of (A) GAPDH mRNA and pre-rRNA by RT-PCR, (B) rRNA by indirect immunofluorescence staining and flow cytometry using anti-rRNA primary antibody probe and secondary mouse IgG antibody conjugated to Alexa Fluor 488 (AX488), and (C) expression of proteins involved in rRNA biogenesis.

Figure 7.

Analysis of CD138-positive cells from the bone marrow of four patients with MM. Cells were isolated, exposed to the indicated concentrations of Clo, gemcitabine Gem, or both for 48 hours and analyzed using Western blot for levels of γ -H2AX and p53. Patient (Pt) number is indicated.

Figure 8.
Suggested mechanism of the synergistic cytotoxicity of Gem and Clo toward multiple myeloma cells.

Cell lines used in this study

Table 1

Cell line	Disease	Cytogenetic karyotype	Unique gene alteration	P53 status
NCI-H929	Myeloma (IgA)	Hypodiploid, 16% polyploidy; 45 (43-46)<2n>X, -X, +8,-1-, -13, +mar, dup(1)(q11q25), del(1)(p11p25), del(6)(q25), Der(8)t(8; ?)(q23; ?)(q23; ?)X2, del(9)(p13), del(12)(p12)	MYC rearrangement	Wild type
MM.1R	Multiple myeloma (IgA)	44, XX, -8, -13, -14, -16, -21, del(1)(p13p22), t(2; ?)(q37; ?), t(3; ?)(p25; ?), t(6; ?)(q22; ?), t(12;14)(q24;q32), +der(8)t(9;13)(q21;q22), +der(16)t(8;16)(p21.1;q12), +der(21)t(1;21)(q12;p13), t(14;16)(q32;q23)	P18/NK4 C deletion	Wild type
RPMI 8226	Multiple myeloma (IgG)	Flat-modulated hypodiploid, 7.5% polyploidy; 62-67<3n>XXY, -1, +5, +6, +7, -8, -9, -10, +11, -12, -13, -14, +16, +18, -19, +21, -22, der(1)t(1;1)(p36.3;q11-12), add(3)(q27-29)X2, del(4)(p14p15.4), der(5;6)(q10p10)X1-2, del(5)(q32), del(6)(q15), add(9)(p24), add(11)(p15), add(11)(q25), der(14)t(1;14)((q11-12;q32)X2, del(15)(q11.2q14)X2, add(16)(q23-24), der(17)t(? 8;17)(q21.2;q25), del(22)(q13.2); FISH: t(1;14)(p13;q32), t(16;22)(q23;q11)	P16/NK4 A methylation	Mutant
U266B1	Myeloma (IgE)	Hypodiploid, 6.5% polyploidy; 44 (40-46)<2n>XY, -8, -10, -13, -15, +2mar, t(1;11)(p33;q13), add(3)(q27), t(4;11)(q21;q23), add(7)(q32), add(8)(q24), add(9)(q34), add(10)(p14), add(14)(p11), add(17)(p11), add(18)(p12), der(22)t(15;22)(q21;q13); SKY: der(1)t(1;3), der(3)t(3;16), der(8)t(1;8;11), der(8)t(7;8), der(11)t(1;8;11), der(13)t(2;13); ins(14;10), t(1;13)(?;q14.2)	P16/NK4 A methylation	Mutant

From Drexler HG. The leukemia-lymphoma cell line factsbook. San Diego: Academic press; 2001.